Chapter 9
Chemical Analysis and Characterization of Biomass for Biorefineries

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Abstract The aim of this chapter is to offer different chemical analyses and characterization options for researchers or whoever is looking for an appropriate methodology to analyze results obtained in laboratory tests, especially assuming the challenge to find the best process to achieve bio-products under biorefinery concept. In this way, the information provided will be very useful to evaluate the results and moreover, to improve the research process. That is the reason why analytical techniques to characterize different lignocellulosic biomass are described with detailed data about its principles and methodology, emphasizing either physical or chemical protocols that are followed normally in research laboratories. Taking into account that lignin, cellulose, and hemicelluloses are the principal compounds of these kinds of raw materials, which in general are residues, the information is emphasized with that target of analysis. Nevertheless, as it is possible to obtain a lot of bio-products from biomass, like sugars, alcohols, aromatics, biopolymers and so on, other analytical methods are included.

Keywords Gravimetric analysis • Compositional analysis • Chemical characterization

9.1 Generalities

9.1.1 Types of Biomass

Biomass is all organic matter produced by the process of photosynthesis where many molecules are synthesized by sunlight. These molecules fulfill a specific function within the plant and denote high contents of chemical energy. Every year, about

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2.2 x 10^{11} dry tons of biomass (Oliva Domínguez 2015) is produced and can supply the demand for global energy, overtaking it about 9 times. It can be categorized according to its origin (Ballesteros et al. 2001) in *natural, residual*, and from *energy crops* as described below.

- Natural biomass: refers to biomass that is generated naturally in each ecosystem. Because it has not undergone any transformation and in order to maintain the habitats where it grows, this type of biomass is not considered suitable to be harnessed for energy or industrial purposes.
- Residual biomass: consists of organic residues generated after natural biomass is transformed. It is classified according to its origin as follows:
  - Agricultural residues such as those generated from *woody and herbaceous* crops, as residues obtained from periodic and annual pruning of coffee and sugar cane, cereal crops (straw, leaves, seed husks, etc.), cane (leaves and tops), palm (leaves, racemes, husks, etc.), hay, rice (straw, husks), and corn (stem, leaves, husk, silk, and tassel), among many others.
  - Forest residues arising from treatments, interventions, and/or forest improvements. In this category, forest residues that can be highlighted are pruning residues, commercial trees with imperfections or trees that need to be cut down that are close to fire zones or that are ill. Also, sawdust and other residues from the production of wood tables.
  - Agroindustrial residues: are the ones originated from papermaking (pulp, process residues), juices (husks, pulp), sugar (bagasse), and from oil extraction (fibers, palm seeds), etc. Household waste is also relevant, especially the ones generated in food storage centers, market places, and administrative facilities of companies, where meaningful quantities of paper waste are generated.
- Biomass energy crops: in this category is the biomass that comes from crops whose purpose is its use for energy production. Two subdivisions can be highlighted within this category: woody crops and fast-growing annual crops of vegetables (Oliva Domínguez 2015).

The biomass type with the greatest potential of use corresponds to the residual origin. This material is a source of energy and can be employed in the field of building materials and in the pharmaceutical, cosmetology, nutraceutical, food, biofuels, and chemical industry. Unlike oil, this biomass can be found everywhere in the world where the conditions for its production are given; it is renewable and has high contents of oxygen.

9.1.2 Biomass Composition

Biomass forms different structures within a plant: roots, stems, leaves, flowers, and fruits. Each of these structures meets different functions in the plant and therefore, the composition is very heterogeneous. They also change between species and

within the same plant and are composed primarily by water, soluble substances (sugars, organic acids), pectin, fiber, lipids, and proteins. The heterogeneity is seen for example, in the study by Cardoen et al (2015), who evaluated the composition of carbon, hydrogen, oxygen, and nitrogen from residues of more than 22 species of plants grown in India. Table 9.1 shows the significant differences in composition between species and between components of the same plant. It is noted how the carbon content in these biomasses is around 30.4 % for corn stalks, mustard and onion and 52.3 % for coconut husk. The next component in greater composition is oxygen. The oily substances from coconut and peanut have a higher content of this component with 63.5 % and 59.9 %, respectively. In the lower range, water hyacinth (34 %) and rice husk (35.9 %) are found. Hydrogen is between 2 and 8.8 %. Other features that may affect this characterization in addition to the ones already stated are the growing conditions, plant growth, soil and agro-climatic characteristics, age,

Table 9.1 Moisture content and elemental analysis of several biomass samples

Crop name (s)	Residue name	Moisture content at in harvest/ production %)	Moisture content in dried air (%)	C (%)	H (%)	O (%)	N (%)
Banana	Leaves, pseudostems						
Banana	Peels	84					1.06
Cabbage	Stem and leaves	85					
Chickpea	Stalks	20	10				0.80
Chickpea Coconut	Husk Fronds	20	10	48.6[a]	5.8[a]	42.4[a]	0.80
Coconut	Husk	20	10				
Coconut	Shell	20	4.4	52.3	6.6	39.5	0.30
Coconut	Meal/oil cake	7		15.9	2.3	63.5	0.50
Coconut	Coir pith			50.3	5.1	39.6	0.45
Cotton	Stalks	20	10	41.5	6.2	47.5	1.81
Cotton	Hull/Boll shell		7	50.4	8.4	39.8	1.40
Cotton Cotton	Gin trash Meal/oil cake	5		42.7	6.1	49.5	0.18 6.45[b]
Eggplant	Stalks	85					
Groundnut	Stalks	30	10				1.60
Groundnut	Shell	30	8	33.9	2.0	59.9	1.10
Groundnut	Meal/oil cake	10					8.00
Maize/corn	Stover	60	10	46.9	5.5	45.0	0.56
Maize/corn	Cobs	50	10	41.4	6.0	51.3	0.14
Maize/corn Mango	Corn fiber/grain hull Pruning wood Pruning wood	50		47.4[a]	6.1	44.4	0.60
Mango	Peels	80		46.4[a]	5.8[a]	43.1[a]	

(continued)

Table 9.1 (continued)

Crop name (s)	Residue name	Moisture content at in harvest/ production %)	Moisture content in dried air (%)	C (%)	H (%)	O (%)	N (%)
Mango	Seed	45	13	47.8[a]	5.8[a]	43.1[a]	0.85[b]
Mango	Meal/oilcake	10					0.96[b]
Mustard	Stalks	20	10	33.7	3.9		0.67
Mustard	Seedpod	20	5.6	44.3	8.8	43.0	0.38
Mustard	Meal/oilcake e	10		50.2	6.9		5.05
Onion	Stalks	85					
Paddy/rice	Straw	30	10	36.0	5.3		0.70
Paddy/rice	Husk	30	10	36.4	4.9	35.9	0.59
Paddy/rice Paddy/rice	Bran De-oiled bran			38.9	5.1	36.8	0.55 2.88[b]
Pearl millet	Stalks	30	10				0.70
Pearl millet	Cobs	30	10				0.70
Pearl millet	Husk	30	10				0.70
Pigeon pea	Stalks	20	10	38.1	6.2	53.8	1.01
Pigeon pea	Husk	20	10				0.80
Potato	Stalks	60					1.90
Sorghum	Stalks	30	10				0.77
Sorghum	Cobs	30	10				0.77
Sorghum	Husk	30	10				0.77
Soybean	Stalks	50	10				0.80
Soybean	Husk	9	6.3	43.1	6.4	44.5	0.80
Soybean	Meal/oilcake	11					7.60[b]
Sugarcane	Tops and leaves	60	10	39.8	5.6	46.8	1.70
Sugarcane	Bagasse	50		48.6	5.9	42.8	0.16
Sugarcane	Depithed bagasse	50		46.1	6.5	46.0	0.41
Sugarcane	Press mud/filter cake			30.4[a]	3.8[a]	28.7[a]	2.00
Sugarcane Cassava Cassava	Molasses Peels Fibrous residue/ bagasse	20.6 75	11.2				0.51[b] 0.84[b] 0.25[b]
Cassava	Stalks/hay		10				1.90
Tomato	Stems	85					4.80
Water hyacinth Agua de jacinto	Whole	90		40.3	4.6	34.0	1.51
Wheat	Straw	20	10	44.9	5.5	41.8	0.44
Wheat	Chaff	20	10				
Wheat	Bran	20	10				

Source: Adapted from Cardoen et al. (2015)

but also the techniques used in this quantification of the different components (Szcrbowski et al. 2014).

It is important to understand how the biomass is chemically constituted in order to set the techniques for its chemical characterization. Biomass consists primarily of cells, which have a cell wall containing the cytoplasm with its organelles and nuclei. The cell wall has primary swellings (primary wall) or secondary (secondary wall) depending on the type of structure that is forming. The primary is rich in hemicellulose and pectin with some cellulose and lignin. Instead, the secondary are richer in cellulose and lignin. The cement that binds a cell with another in the middle lamella is pectin.

9.1.2.1 Cellulose

Cellulose is a lineal carbohydrate with crystalline structure and a molecular formula $(C_6H_{10}O_5)_n$ (in ranges from 1000 to 5000) and is composed of β-D-glucose units in pyranose shape, which can be broken into glucose units by hydrolysis (Bian et al. 2014). Its structure is organized by hydrogen bonding between hydroxyl groups of different juxtaposed glucose chains offering impermeability to water, making it insoluble in this solvent and thus, forming compact fibers that make up the wall structure in plant cells which becomes into a relevant property to this polymer. The distribution of these fibers may be different at the levels of the secondary wall favoring it with great strength. Since this polymer is the major component of the cell wall in vascular plants (Bhattacharya et al. 2008), it becomes the most abundant molecule in plants. Thanks to this abundance, this molecule is in a lot of manufactured products like paper, building, and textile materials made form fibrous plants such as cotton and linen.

9.1.2.2 Hemicellulose

It is a heterogeneous branched polymer with molecular formula $(C_6H_{10}O_5)_n$ or $(C_5H_8O_4)_n$ and is present in the cell walls with amorphous or paracrystalline distribution. The structure contains amorphous polymers, such as pentoses, hexoses, deoxyhexoses, and uronic acids, which form a branched straight chain. In addition, other polysaccharides like xylan, arabinoxylans, galactomannans, glucuroarabinoxilanes, glucomannan, and xyloglucan can be found. With regards to the structural characteristics of this polymer, the following can be mentioned (Martín-Lara 2008):

- Hemicellulose consists of a sugar chain linked by β-(1-4) bonds assuming the shape of a backbone with branches of one or two types of sugar (xylan, mannan, galactan, glucan, glucuronoxylan, galactoglucomannan, etc.).
- It cannot generate aggregates unlike cellulose due to the structure. Yet, hemicellulose can join the cellulose chains by building hydrogen bonds.
- It degrades easily by having short chain branching (Fengel and Wegener 1984).

– Additionally, hemicellulose is in charge of binding lignin with cellulose and has little resistance to hydrolysis or heat.
– Following cellulose, this polymer is found most abundantly in the cell wall of plants.

9.1.2.3 Lignin

Lignin is a tridimensional (Higuchi 1985; Boerjan et al. 2003) aromatic polymer with molecular formula ($C_9H_{10}O_2(OCH_3)_n$). It is formed by dehydrogenation of enzymes from the phenylpropilic alcohols (coumaryl, coniferyl, and sinapyl) first, and then, by polymerization causing different types of distribution among its components and determining that lignin has no single structure due to lack of enzyme control and free radicals reaction that react together (Turrel and Fisher 1942). In cell growth, this rigid and hydrophobic substance is adhered to the cell wall by filing the empty spaces between the cellulose fibrils joining covalently to the matrix of polysaccharides. Lignin is part of the secondary cell walls of plants generating a resistant structure difficult to degrade. This polymer provides support to the structure of plant tissues and due to the chemical composition, protects against humidity and/or attack of atmospheric organisms and mechanical stress. It also acts as a type of fiber binder (Sarkanen and Ludwig 1971)

After cellulose and hemicellulose, lignin is the non-carbohydrate component found in higher quantities on cell walls and on the surface (Buranov and Mazza 2008).

9.1.2.4 Pectin

Pectic substances are linear molecules of galacturonic acid (α-D-1,4-galacturonan), with insertions at certain intervals of rhamnose units (α-L rhamnosil residue), where side chains emerge rich in neutral sugars (hairy regions). These side chains are attached by glycosidic linkages to the carbon atoms 3 and 4 of rhamnose units and to the atoms 2 and 3 of the galacturonic acid units. The predominant sugars depend on the structure of the pectin where the most important are D-galactose, L-arabinose, and xylose (Pilnik and Voragen 1993). Pectin contributes to offer mechanical strength, porosity, adhesion, and rigidity to the cell wall. They are mostly found in the middle lamella and the primary walls of upper plants.

9.1.2.5 Other Components

Other components are constituted of proteins, ash, lipids and solubles (sugars and organic acids), as well as waxes and gums.

9.2 Chemical Analysis of the Major Components of the Cell Wall

As mentioned in Sect. 9.1, the chemical characterization of biomass is complex due to the factors that affect its structure: type of tissue within plants, age of crop, type of soil, variety, agro-climatic growing conditions, etc. Szcrbowski et al. (2014) observed these factors thoroughly on sugar cane bagasse and cane straw. They found that tilage, soil, and weather factors are probably the main reasons why there is a wide range of differences in reporting the chemical composition of both residues. Additional methods used in the characterization of biomass also influence as follows: (a) the use of free-contaminant extractives for lignin analysis, (b) the degree of removal of extractives during treatment with different solvents, (c) proper quantification of minor components, such as pectin and proteins, (d) the use of HPLC columns with different resolutions for monosaccharides analysis, and (e) applying suitable hydrolysis factors to consider sugar losses due to dehydration. Therefore, there is no 100 % method approved by the scientific community to study its composition. The methods that have been most accepted are the following.

9.2.1 Methods for Extractives Extraction

9.2.1.1 Characterization of Soluble Extractives in Water

There are several methods to extract water-soluble compounds, among them are:

- Soxhlet method.
- Liquid–liquid extraction (Golander 2011).
- Cold water-solubility method (ASTM D1110-84 2013).
- Hot water-solubility method (Szcrbowski et al. 2014).
- Ultrasound-assisted extraction (Luque de Castro and García-Ayuso 1998).
- Microwave-assisted extraction (Golander 2011).

Soxhlet has been the standard technique for over a century and is referent to be compared against other modern extraction techniques (Luque de Castro and Priego-Capote 2010). Furthermore, this method has various advantages since the sample is contacted repeatedly with fresh solvent portions, which contributes to shift the equilibrium of transfer. On the other hand, the system temperature remains relatively high because the heat applied to distillation reaches the extraction unit. Also, it is not necessary to filter at the end of extraction. The equipment is basic and thus, economic. It is a very simple methodology that requires little specialized training and has the ability to extract more compounds from the sample than the majority of modern methods (microwave extraction for example) (Luque de Castro and García-Ayuso 1998).

Principles

Soxhlet extraction is based on the following steps: (1) Solvent-location in a flask. (2) Solvent boiling which is evaporated to a reflux condenser. (3) Formation of condensate falling on a vessel containing a porous cartridge with the sample therein. (4) Solvent level rise covering the cartridge until a point that reflux occurs. (5) The process is repeated as necessary until the sample is exhausted.

The technique relies on the extraction of water-soluble compounds (solvent used). Soxhlet is considered a continues–discontinuous hybrid technique since the solvent is involved in several cycles and each complete cycle is appreciated as a bath type system. At the same time, the solvent offers the system a continuous character when it recirculates. On the other hand, because water is in prolonged contact with the sample, it allows the extraction of compounds that solubilize therein like secondary metabolites and those that store energy which are critical for photosynthesis and plant growth (sucrose and pectin) (Szcrbowski et al. 2014). In addition, sugars like glucose and fructose can be found. The fact that these compounds are extracted is attributed to the polar character for both the extractant and the metabolites. An analytical technique well known as Fourier transform infrared spectroscopy (FTIR) identifies such metabolites extracted due to the pronounced vibration band corresponding to the molecular hydroxyl groups (–OH). This technique will be analyzed in more detail in Sect. 9.2.2.6. For spectroscopic analysis see Sect. 9.2.2.6.

Methodology

The procedure for performing compound extraction by Soxhlet method takes as reference the protocol "Technical Report NREL/TP-510-42619 January 2008: Determination of Extractives in Biomass" (Sluiter et al. 2008a) and is as follows.

The ground sample must consist of 8–20 g, obtained in such a way that it is representative of the entire batch of material. It is required at least 8 g of the sample to do the complete analysis. The sample must be divided into smaller fragments with certain periodicity. In the case of dry lignocellulosic biomass, the material must be ground until it passes through the mesh 40 (Tappi T 264 om-82).

Preparing Sample for Extraction

1. The moisture content of a sample of biomass can change quickly when it is exposed to air. Therefore, samples have to be weighed in order to determine the total amount of solids (Sect. 9.4), and also extractives at the same time and thus, prevent errors due to humidity changes.
2. Dry the flat bottom ball in an oven at 105 ± 5 °C for a minimum of 12 h. Then, it is removed and allowed to adapt to room temperature by placing it in a desiccator. After that, boiling stones are added and the weight of the ball is registered.
3. Add 2–10 g of the sample to an extraction cartridge (cellulose thimble) that has been weighed and tared previously. Record the weight to the nearest 0.1 mg. The amount of sample needed depends on the bulk density of the biomass.

The height of the biomass present in the cartridge must not exceed the height of the siphon of the Soxhlet extraction tube. If the height of the biomass surpasses the height of the siphon, the extraction will be incomplete.

4. Add solvent (water) to fill the flask until half of its capacity. Insert the cartridge into the Soxhlet extraction tube. Assemble the equipment and turn on. Adjust the heating mantle to provide a minimum of 4–5 siphon cycles per hour.
5. Reflux for 6–24 h. The reflux time required will depend on the rate of elimination of the compounds of interest, the temperature of the capacitors, and the flow rate in the siphon. When the sample of biomass is exhausted, turn off the equipment. Dry the cartridge with the sample. The weight difference between the initial and the final weight divided by the initial weight of biomass gives the percentage of solubles extracted in water. Note that the weight of the dry cartridge has to be subtracted to the final weight of the biomass.

9.2.1.2 Analysis of Soluble Extractives in Solvents

There are diverse methods to extract soluble compounds in solvents similar to the ones mentioned previously (Sect. 9.2.1.1), where the most relevant are the following:

– Soxhlet method.
– Liquid–Liquid extraction.
– Ultrasound-assisted extraction.
– Microwave-assisted extraction.

In this case, Soxhlet is also the most common technique applied in the extraction of compound solubles in solvents because it has great resilience of solutes and simpler instrumentation. The solvent and sample are in intimate and repeated contact, so that the extraction improves greatly due to the constant use of a clean solvent, ensuring optimal performance. The organic solvent is evaporated leaving the solutes upon completion of the process.

Principles

The principle of the method is the same as is used to extract water-soluble compounds. In this case, what changes is the use of organic solvents. Soxhlet extraction has the following steps: (1) location of the solvent in the flask. (2) Solvent boiling, which evaporates to a reflux condenser. (3) Formation of condensate falling on a vessel containing a porous cartridge with the sample therein. (4) The level of the solvent rises covering the cartridge to a point that reflux occurs by the siphon, which is done with solvent extracted by the reflux sidearm system. (5) The process is repeated as many times as necessary until the sample is exhausted.

Table 9.2 Extractant used to solubilize polar and nonpolar compounds

Raw material	Extractant	Conditions	Results (%)	References
Bagasse	Ethyl ether Dichloromethane Ethanol:toluene 1:2 Ethanol 95 % v/v Water	Soxhlet extraction. Reflux for 1 h	9.0 1.0 12.0 2.2 11.5	Szcrbowski et al. (2014))
Bagasse	Hexane	Soxhlet extraction. Reflux for 4 h	0.53	Attard et al (2015)
Bagasse	Water	Soxhlet extraction. Reflux for 3 h	1.3	Del Río et al (2015)
Straw	Ethyl ether Dichloromethane Ethanol:toluene 1:2 Ethanol 95 % v/v Water	Soxhlet extraction. Reflux for 1 h	11.5 1.5 12.2 2.5 36.0	Szcrbowski et al. (2014))
Straw	Water	Soxhlet extraction. Reflux for 3 h	2.1	Del Río et al (2015)
Leaves	Hexane	Soxhlet extraction. Reflux for 4 h	1.60	Attard et al. (2015)
Peel	Hexane	Soxhlet extraction. Reflux for 4 h	0.8	Attard et al (2015)
Chlorella vulgaris (microalgae)	Cloroform:methanol 1:2	Soxhlet extraction. Reflux for 6 h	20.22	Pérez and Quishpi (2014)
Olive Pomace	*n*-Hexane	Soxhlet extraction. Reflux for 4 h	2.14	García Sánchez et al (2005)
Olives	*n*-Hexane	Soxhlet extraction. Reflux for 4 h	24.58	García Sánchez et al. (2005)

Results in % m/m

Various organic solvents have been used as shown in Table 9.2. It can be seen as the nonpolar compounds are extracted by ethanol/toluene, hexane, and chloroform-methanol. The latter mixture is the most widely applied, because it has the ability to extract lipids, fatty acids, and sterols. This mixture combines the ability from ethanol, which consists in penetrating the tissue with the power of chloroform in order to dissolve fat.

Methodology

The reference used is the protocol "Technical Report NREL/TP-510-42619 January 2008: Determination of Extractives in Biomass" (Sluiter et al. 2008a). The most specific method for performing extractions of compounds by Soxhlet is shown below. However, in characterization of water-soluble extractives the methodology for Soxhlet is explained in detail (Fig. 9.1).

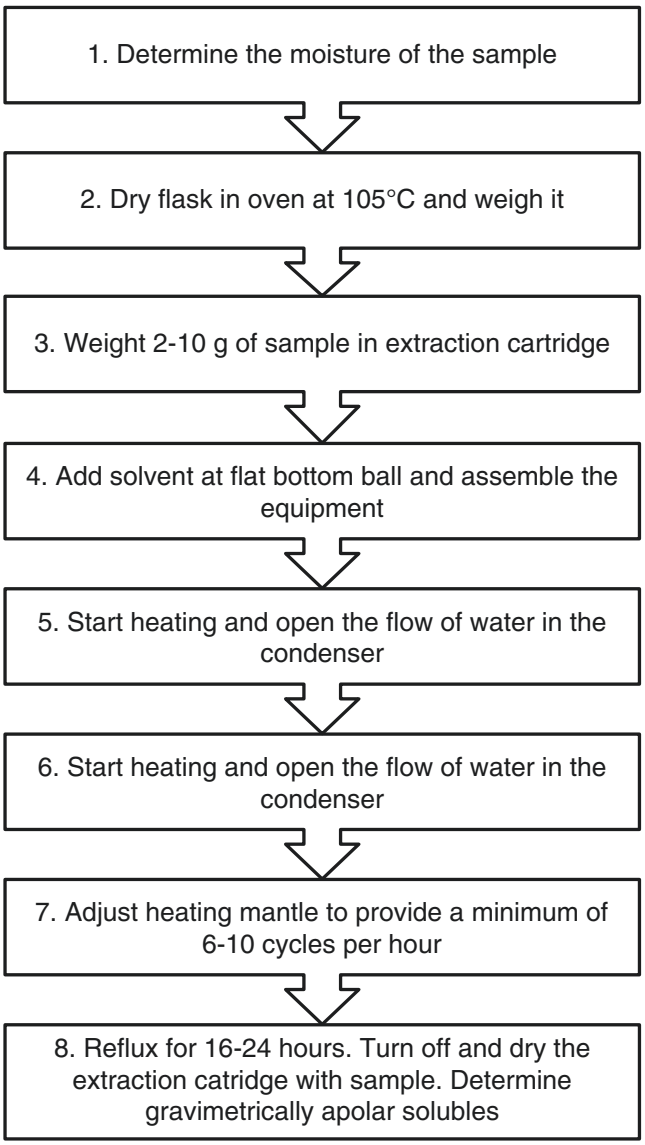

Fig. 9.1 Flow chart for water-soluble extraction by Soxhlet method

9.2.2 Analysis of Polysaccharides (Cellulose and Hemicellulose)

Hemicellulosic and cellulosic chains are joined together by internal attractive forces through the carboxyl and hydroxyl functional groups of the macromolecules of cellulose and hemicelluloses (Cataño Rueda 2009). The characterization of these polysaccharides is done through various chemical methods already established as mentioned bellow.

9.2.2.1 Van Soest Method

Principles

The Van Soest method is widely used because it measures separately cellulose, hemicellulose, and lignin. It is based on the digestion of a sample in a neutral detergent solution. The soluble fraction is cell content (CC) and the residual fraction is called neutral detergent fiber (NDF), which are cell walls. Once DC and NDF are separated, the solid residue is treated with a solution of acid detergent composed by bromide trimethyl-acetyl-ammonium in 1 mol/L sulfuric acid. The intention of this treatment is that hemicellulose can be solubilized while in the insoluble fraction, called acid detergent fiber (ADF), cellulose, lignin, and cutin remain. Once NDF is obtained, the percentage of cellulose is determined with sulfuric acid treatment. The final fractions are, on the one hand, cellulose (that is extracted), and on the other, lignin-cutin-ash residue. This residue is treated to obtain lignin employing permanganate treatment. Cutin-mineral residues that remain are incinerated in order to obtain the percentage of cutin.

Methodology

The methodology described below is based on AOAC 973.18 (1995) and AOAC 2002.04 (2002). Both the analysis NDF and ADF can be performed directly, or can be performed sequentially. The sequential method has the advantage of obtaining all the results from a single sample, although the values obtained for the second analysis may not be exactly comparable to those achieved with the same analysis done directly.

Procedure

Figure 9.2 shows the components in a Fibertest.

It is important to illustrate the way to prepare the reactives in order to perform the procedure:

ND: Neutral detergent

Sodium lauryl sulfate	30 g/L
Ethylene diamine tetra-acetic acid (EDTA) anhydrous	18.6 g/L
Borate decahydrate sodium	6.8 g/L
Disodium hydrogen phosphate anhydrous	4.6 g/L
2-Ethoxyethanol	10 mL
Distilled water	Up to 1000 mL

Mix sodium borate and EDTA in a beaker of 2 L. Add distilled water, heat to dissolve. Add the lauryl sulfate. Weigh disodium phosphate and add distilled water in another flask, heat the solution until it is completely dissolved. Mix the solutions, add 2-ethoxyethanol to limit foaming. Check the pH value is between 6.9 and 7.1.

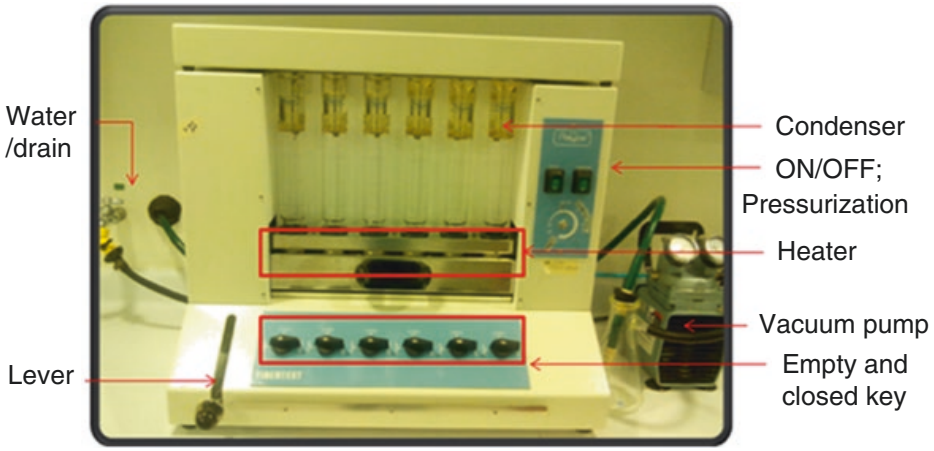

Fig. 9.2 Digestor for polysaccharides analysis

ADS: Acid detergent

Cetyl trimethyl ammonium bromide	20 g/L
Sulfuric acid 1 mol/L	1 L
Dissolve 20 g of cetyl trimethyl ammonium bromide in the sulfuric acid	1 mol/L

Amylase solution

Alpha-amylase (density: 1.13 g/mL)	2 g
Ethoxyethanol	10 mL
Distilled water	To fulfill 1 L

Dissolve 2 g of amylase in distilled water and adjust with ethoxyethanol. Keep the solution at 5 °C.

Sulfuric acid at 72 %

Dilute 750 mL of acid (96 % concentration) in water slowly to make 1 l of solution.

Note: The reaction is strongly exothermic, so it is recommended to be in a cold water bath.

(a) Sample preparation: Remove the solubles of the sample (see Sects. 9.1.1 and 9.1.2). Use the ethanol to extract apolar solubles as recommended by the NREL (Sluiter et al. 2008c). Dry at 60 ± 3 °C for 18 h and determine the moisture of the sample.
(b) Crucible preparation: wash and let the crucible submerged in a sulfochromic solution for 8 h, then rinse and dry. Weigh the dried crucible with a smaller difference of 0.1 % (weight = *P*0).
(c) Weigh 1 g of the sample in the crucible with a minor difference of 0.1 % (taking weight as *E*).
(d) Fiber digestion: to locate the crucible in fibertest: lift the lever and locate the crucible in the heating zone below the glass column. Then, pull down the lever. Verify the crucible is pressed to the system.
(e) Open the water stopcock that feeds the cooling system over the columns avoiding high water pressure.

(f) Add 50 mL of neutral detergent solution (NDS) at the top of the columns, turn the equipment on and set the temperature of the heater. Once boiling point is reached, count 30 min.

Note: The boiling should not be violent to prevent the material sticking up on the walls of the columns.

(g) After 30 min, add another 50 mL of neutral detergent solution with 2 mL of alpha-amylase to solubilize the starch content in the sample. Boil for additional 30 min.
(h) After the heating is stopped, the keys are placed in a position to filter vacuum. The equipment must be connected to a vacuum and drain systems. Several washes with hot distilled water and acetone are made until foam is unnoticeable.
(i) Lift lever up to remove the crucible with the insoluble material. Heat the sample on the stove at 105 °C for 8 h or until constant weight. Then, cool the crucible with the sample in a desiccator for 20 min and weigh with a weight difference less than 0.1 % ($P1$).
(j) Place the crucible back on the fibertest and adjust to the columns.
(k) Add 50 ml of acid detergent solution (ADS) at the top of the columns, heat to boiling point, and count 1 h when boiling point is reached.
(l) Stop heating and wash with hot distilled water repeatedly until foaming runs out.
(m) Remove the crucible. Dry in the oven at 100 °C for 8 h or until constant weight. Then, place the sample in the desiccator for 20 min and weigh for a weight difference of less than 0.1 % ($P2$).
(n) After that, place the crucible in a glass container and add sulfuric acid at 72 % v/v to the sample to cover it. Shake and leave for 3 h.

Note: Do not allow a decrease on the level of sulfuric acid.

- Then, rinse with hot distilled water to remove sulfuric acid. A vacuum system can be used to facilitate washing.
- Heat the crucible with the sample at 100 °C in an oven for 8 h or until constant weight, followed by placing it in the desiccator for 20 min and weigh to obtain weight $P3$ with a minor difference of 0.1 %.
- In order to mineralize the sample, the crucible is carried muffle at 550 °C for 4 h followed by an hour in the oven at 100 °C.
- Finally, cool the sample down in the desiccator for 20 min and weigh to obtain the weight $P4$ with a minor difference of 0.1 %.

Calculi

$$\text{NFD} = \frac{P1 - P4}{E} \times 100 \tag{9.1}$$

$$\text{ADF} = \frac{P2 - P4}{E} \times 100 \tag{9.2}$$

$$\%\,\text{Hemi} = \frac{P1 - P2}{E} \times 100 \tag{9.3}$$

$$\%\,\text{Cel} = \frac{P2 - P3}{E} \times 100 \tag{9.4}$$

$$\%\,\text{Lig} = \frac{P3 - P4}{E} \times 100 \tag{9.5}$$

$$\%\,\text{Ce} = \frac{P4 - P0}{E} \times 100 \tag{9.6}$$

Note: NDF is neutral detergent fiber; FDA is acid detergent fiber; Hem is the hemicellulose fraction; Cel is cellulose; Lig, lignin; Ce, ash; and *E*, corresponds to the initial weight of the sample.

9.2.2.2 NREL Method (According to Sluiter et al. 2008a)

Principles

This protocol uses sulfuric acid hydrolysis in order to dissociate the polymeric forms of carbohydrates present in biomass into monomeric subunits. The function of the sulfuric acid is to hydrolyze glycosidic bonds to give monosaccharides. After the Oligo or monomers are quantified by high-performance liquid chromatography (HPLC). The HPLC method will be presented in Sect. 9.2.2.5.

9.2.2.3 Methodology

Sample Preparation for Analysis and Hydrolysis

1. Place a suitable number of filtering crucibles in muffle at 575 ± 25 °C for 4 h minimum. Remove the muffle crucibles and pass them directly to a desiccator. Allow to cool for about 1 h. Weigh crucibles and record. It is important to keep the melting pots in a specific order, if they are not marked properly. Do not mark the bottom of the pot, as this can prevent filtration.
2. Weigh 300.0 ± 10.0 mg of the sample or standard of quality control (standard sugars) in a pressure tube. Record the weight to the nearest 0.1 mg. This standard of quality control (standard sugar) ensures hydrolysis conditions are suitable for carrying out this process. Label the pressure tube with a permanent marker. Each sample is tested in duplicate.
3. Add 3.00 ± 0.01 mL (or 4.92 ± 0.01 g) of 72 % sulfuric acid to each pressure tube. Use a Teflon bar to stir the mixture for 1 min.
4. Place the pressure tube in a water bath at 30 ± 3 °C and incubate the sample for 60 ± 5 min. The sample is stirred every 5–10 min using the stirring bar, and

without removing the sample from the bath. Agitation is essential to ensure contact between the acid and the sample particles and thus, ensuring uniform hydrolysis.

5. After 60 min, remove the tubes from the water bath. Dilute acid at a concentration of 4 % by the addition of 84.00 ± 0.04 mL of deionized water. Dilution may also be done by adding 84.00 ± 0.04 g of purified water using a precision scale of 0.01 g. Cap the tube and shake in a vortex to eliminate phase separation between the layers of high and low acid concentration.

   Note: 86.73 mL will be the volume of sulfuric acid solution at 4 %.

6. Prepare a set of sugar recovery standards (SRS) that be will used during remaining process to correct losses due to the destruction of sugars during dilute acid hydrolysis. The SRS should include D-(+) glucose, D-(+) xylose, D-(+) galactose, L-(+) arabinose, and D-(+) mannose. Sugar concentrations in the SRS should be chosen to resemble more closely at concentrations of sugars in the sample tested. Weigh the necessary amounts of each sugar, with accuracy of 0.1 mg, and add 10,0 mL of deionized water. Add 348 µL of sulfuric acid at 72 %. Transfer SRS to a pressure tube and cover.

A fresh solution of SRS is not required for each analysis. Large quantities of SRS can be prepared: the solution is filtered through 0.2 µm filters and dispensed in aliquots of 10.0 mL storing it in sealed and labeled containers. They can be stored in a freezer. When required, thaw and shake the frozen SRS before use.

7. Place the tubes in a safety rack and then in the autoclave. Autoclave the sealed samples and sugar recovery standards for one hour at 121 °C. Allow the hydrolyzate to slowly cool down at room temperature before removing caps from pressure tubes after completion of the autoclave cycle.

Note: To deepen in the analysis of the hydrolyzed sugars, see Sect. 9.2.2.5. If the hydrolyzate obtained determines insoluble and soluble lignin in acid, take only 1 mL for characterization by HPLC.

Sample Analysis of Acid Insoluble Lignin (According to Sluiter et al. 2008a)

1. Vacuum filter the previous hydrolysis solution through preweighed filtering crucibles. Capture filtering in a filter flask.
2. Transfer an aliquot of about 50 mL in a sample storage bottle. This sample is used to determine acid soluble lignin and carbohydrates, and acetyl if necessary. Determining acid soluble lignin must be done within 6 h following the completion of the hydrolysis. If the hydrolysis liquor should be stored, this must be done in a refrigerator for up to 2 weeks. It is important to collect the aliquot of liquor before proceeding to the next step.
3. In order to transfer all solids remaining in the pressure tube quantitatively to the filter crucible, use deionized water. Rinse the solids with a minimum of 50 mL fresh deionized water. Hot deionized water can be used instead of water at room temperature to reduce filtration time.
4. Dry the crucible with the acid insoluble residue at 105 ± 3 °C to constant weight, for at least 4 h.

5. Remove samples from oven and let cool in a desiccator. Record the weight of the dry residue nearest to 0.1 mg.
6. Place the crucibles with the residues in the muffle at 575 ± 25 °C for 24 ± 6 h. The muffle can be used with a temperature ramp:
   Programming the muffle furnace temperature:

   - Ramp from room temperature to 105 °C.
   - Temperature remains at 105 °C for 12 min.
   - Ramp to 250 °C at 10 °C/min.
   - Temperature remains at 250 °C for 30 min.
   - Ramp to 575 °C at 20 °C/min.
   - Temperature remains at 575 °C for 180 min.
   - Temperature drops to 105 °C.
   - Temperature remains 105 °C until the samples are removed.

7. Remove the crucible carefully from the oven directly into a desiccator and let cool down for a specific amount of time, which is equal to the initial cooling time of the crucibles when empty. Weigh the crucibles with ashes to the nearest 0.1 mg and record the weight.

Acid-soluble Lignin Sample Analysis

1. Place deionized water or 4 % sulfuric acid in a UV-visible spectrophotometer as a target.
2. After filtration of the hydrolyzate, use an aliquot of liquor hydrolysis obtained during the process. Measure the absorbance of the sample at a suitable wavelength (for bagasse, radiata pine and black poplar, the wavelength recommended is 240 nm; while for corn stover is 320 nm). Dilute sample as necessary for the absorbance is within range 0.7–1.0. Deionized water or 4 % sulfuric acid can be used to dilute the sample, although the same solvent has to be used as the target. Read the absorbance with three decimal places. Analyze each sample at least twice. (This step must be done within 6 h after hydrolysis.)

Calculi

- Calculation of dry weight in oven (ODW)

$$\text{ODW} = \frac{\text{Weight of dry sample} \times \%\text{total solids}}{100} \tag{9.7}$$

- Calculation of the percentage of acid-insoluble residue (AIR) and for acid-insoluble lignin (AIL)

$$\%\text{AIR} = \frac{\text{Weight of crucible with AIR} - \text{Weight of empty crucible}}{\text{ODW of the sample}} \times 100 \tag{9.8}$$

$$\%\mathrm{AIL} = \frac{\begin{array}{l}\text{Weight of crusible with AIR} - \text{Weight of empty crusible} \\ -\left(\left(\text{Weight of crusible with ash}\right) - \text{Weight of empty crusible}\right) \\ -\text{Weight of protein}\end{array}}{\text{ODW of the sample}} \times 100$$

where:

the weight of the protein is the amount of protein present in the acid-insoluble residue. This quantity is necessary for biomasses that contain high amounts of protein.

- Calculation of acid-soluble lignin (ASL)

$$\%\mathrm{ASL} = \frac{\mathrm{UV}_{\mathrm{abs}} \times \mathrm{Volume}_{\mathrm{filtrated}} \times \mathrm{Dilution}}{\varepsilon \times \mathrm{ODW} \times \text{cell length}} \times 100 \tag{9.9}$$

where:

$\mathrm{UV}_{\mathrm{abs}}$ = UV–Vis absorbance of the sample at the appropriate wavelength (see Table 9.3).

$\mathrm{Volume}_{\text{hydrolysis liquor}} = \mathrm{Volume}_{\mathrm{filtrated}} = 86.73$ mL

$$\mathrm{Dilution} = \frac{\mathrm{Volume}_{\mathrm{sample}} + \mathrm{Volume}_{\text{dilution solvent}}}{\mathrm{Volume}_{\mathrm{sample}}} \tag{9.10}$$

$\mathcal{E}$ = absorptivity of biomass to a specific wavelength (see Table 9.3).

ODW = weight of the sample (mg).

Cell length = cm.

Note: The maximum values of λ often contain interference peaks caused by the breakdown of carbohydrates. Recommended wavelength values are chosen to minimize interference.

- Calculi of total lignin

$$\%\mathrm{Lignin} = \%\mathrm{AIL} + \%\mathrm{ASL} \tag{9.11}$$

Table 9.3 Absorptivity constants for measuring acid-soluble lignin according to the types of biomass

Type of biomass	$\lambda_{\max}$ (nm)	$\mathcal{E}$ to $\lambda_{\max}$ (L/g cm)	Recommended wavelength (nm)	Absorptivity to a recommended wavelength (L/g cm)
Pinus Radiata-NIST SRM 8493	198	25	240	12
Bagasse-NIST SRM 8491	198	40	240	25
Corn bagasse	198	55	320	30
Populus deltiodes-NIST SRM 8492	197	60	240	25

9.2.2.4 Godin Method

Godin et al. (2011) developed a new and innovative method that merges the methodology of Van Soest and NREL to analyze structural carbohydrates, cellulose, and hemicellulose in lignocellulosic biomass. In this method, they performed a modification to Van Soest extraction method using only neutral detergent (NDS) to remove all compounds that interfere with sulfuric acid hydrolysis, such as nitrogen and inorganic compounds, chlorophyll, waxes, and other minor compounds. In addition, the extraction is performed with the intention of avoiding interference in the chromatographic quantitation of hemicellulosic and cellulosic monosaccharides. Structural carbohydrates are then submitted to hydrolysis with sulfuric acid to obtain free monosaccharides which are then analyzed by liquid chromatography with Corona charged aerosol detection (LC-CAD).

Methodology

Sample Preparation

Biomass samples are dried in an oven at 60 °C for 72 h. Then, they are passed through a hammer mill to have a size sample of 4 mm, followed by a second milling by disk mill so the sample reaches 1 mm size. Samples are stored at room temperature and protected from light for later use.

1. Analysis for structural carbohydrates by the method of Van Soest modified

Weigh 2 g of dried and ground biomass in a crucible for filtration. Add 100 mL phosphate buffer at pH of 7 with a concentration of 0.1 mM containing 1000 U analytical α-amylase thermostable. The extraction is done in a reflux equipment for 15 min at 90 °C. After the first extraction, the sample in the crucible is vacuum filtered. The withheld solid is submitted to another extraction with 100 mL of neutral detergent solution (NDS) for 1 h at 100 °C. The sample is again vacuum filtered, washed with deionized water and the remaining solid is dried at 40 °C for 72 h. This solid is ground in a laboratory using a mill cooled with cold water.

1. Sulfuric acid hydrolysis.

Weigh 200 mg of dried and ground retained solid in a 100 mL pressure tube and add 3 mL of sulfuric acid at 72 % w/w containing phenol at 0.1 % w/v. Perform washing with nitrogen in the mouth tube, cover and incubate in a water bath for 1 h at 30 °C. Subsequently, dilute H_2SO_4 at 4 % w/w by adding 84 mL of deionized water. Wash again with nitrogen in the mouth tube, cover and incubate in a forced air convection oven for 2 h at 121 °C. Phenol and nitrogen are used to prevent oxidation.

Cool the pressure tube at room temperature before removing cap. The hydrolysis solution is filtered in a filter crucible with a pore size of 40–100 μm; then, both the pressure tube and the filter crucible are washed with deionized water. The filtrate is collected in a 100 mL flask.

Transfer 10 mL of the hydrolyzate filtrate into a 50 mL centrifuge tube and neutralize to pH 7 with solid calcium carbonate. Shake the tube and centrifuge for 5 min at 6000 × *g*. Transfer 1 mL of the supernatant to a 1.5 mL centrifuge tube containing 0.5 mL of acetonitrile to precipitate the residual calcium sulfate. The tube is stirred and centrifuged for 5 min at 6000 × *g*. The supernatant must be filtered through a 0.2 μm filter into a vial for chromatography and then quantified by LC-CAD.

To prevent overestimation of the concentration of monosaccharides due to degradation in acidic media, use a mixture of standard recovery sugars (SRS): the mixture should be prepared in a 10 mL beaker containing sulfuric acid at 72 and 0.1 % w/v phenol. The concentration of the standards should be: 60 mg/mL of D-glucose, 32.5 mg/mL of D-xylose, 2.5 mg/mL L-arabinose, and 3.1 mg/mL of D-galactose. This mixture should receive the same treatment given to biomass. Prepare a target in the same way without biomass.

2. LC-CAD analysis.

The chromatographic run-time was performed by injecting 35 μL of the solutions prepared within a separation module of liquid chromatography. It is advisable to use an analytical column Carbo Sep CHO-682 Pb with a 300 mm × 7.8 mm size ID and particle size 7 μm; and a precolumn Carbo Sep CHO-682Pb LC size of 20 mm × 4 mm ID and particle size 7 μm. Samples are circumvented with deionized water at 80 °C for 30 min with a flow 0.4 mL/min. The charged aerosol detector is set at a maximum current of 50 pA and the gas pressure is 246 kPa.

Calculi

Estimating the content of cellulose, xylan, arabinan, mannan and galactan and is calculated as the total mass of D-glucose, D-xylose, L-arabinose, D-mannose, and D-galactose respectively. The content (g/100 g DM) of a single neutral polysaccharide (NP = cellulose, xylan, arabinan, mannan and galactan) in a given anhydrous sugar is calculated as follows:

$$\mathrm{NP} = \frac{\mathrm{MF} \times \mathrm{CF} \times \mathrm{ND} \times 100}{\mathrm{RF} \times \mathrm{MS} \times \mathrm{DM}} \left(\mathrm{g} / 100\,\mathrm{g\,DM}\right) \tag{9.12}$$

where:

MF = Corresponds to the mass of a given monosaccharide in a 100 mL beaker after acid hydrolysis (in grams).

CF = Monosaccharide mass conversion factor to a polysaccharide residue (0.90 for D-glucose, D-mannose, and D-galactose; 0.88 for D-xylose and L-arabinose).

ND = Ratio of the dry mass of the retained solid after extraction with NDS on the dry mass of the sample before extraction (in g DM/g DM).

MS = Dry mass of the withheld solid used for hydrolysis with sulfuric acid (in grams of humid matter (WM)).

RF = Monosaccharide recovery factor.

DM = dry matter content obtained at 103 °C for 4 h from the sample used for the hydrolysis with sulfuric acid (in g DM/g WM).

Hemicellulose is calculated as the sum of the content of xylan, arabinan, mannan, and galactan. Cellulose + hemicellulose is calculated as the sum of the content of cellulose and hemicellulose.

9.2.2.5 Other Extraction Methods for Polysaccharides

Several methods of chemical pretreatments have been evaluated for their ability to remove lignin and hemicellulose or increasing the permeability of the hemicelluloses–cellulose–lignin matrix. Some methods include treatment with organic solvents, acids, alkali, monoethanolamine, hot water, among others (Foyle et al. 2007), besides the ultrasound and microwave-assisted extraction (Huang et al. 2010). These methods are mainly used to quantify polysaccharides obtained after each extraction based on analytical techniques.

Extraction with Organic, Acid, and Alkali Solvents

Chaa et al. (2008) set the basis for this protocol. Different methods of extraction were mentioned in Sects. 9.2.1.1 and 9.2.1.2. Nevertheless, the method described by Chaa et al. (2008) denotes the advantage of extracting several compounds through the same method, for example, hemicellulose, soluble polysaccharides in water and room temperature, hot-water solubles, solubles in EDTA, NaOH/EtOH solubles and also, solubilize lignin of the biomass.

Methodology

1. Twenty gram of ground biomass is refluxed twice with a mixture of chloroform–methanol (150 mL/150 mL) for 14 h. Then, it is treated with ethanol at room temperature for 2 h and finally with boiling ethanol for 2 h. The insoluble residue in the cell wall that is recovered by filtration with a nylon mesh must be dried at 50 °C for 48 h.
2. The cell wall is treated with stirring sequentially: (a) In water at room temperature for 2 h. (b) With boiling water for 2 h. (c) In Ethylene diamine tetra-acetic acid (EDTA) aqueous at 1 %, pH 6.8 and 80 °C for 4 h. An extraction by filtration must be done at each step that has to be precipitated with 3 volumes of 95 % v/v ethanol, centrifuged, resuspended in water and dialyzed against distilled water at room temperature in order to obtain water-soluble polysaccharides, hot-water soluble polysaccharides, and EDTA soluble polysaccharides.
3. The remaining insoluble residue is then treated with a solution of 1 % NaOH and 70 % ethanol at 80 °C for 2 h to solubilize lignin. Weight loss is defined as NaOH EtOH-lignin. NaOH-EtOH soluble polysaccharides are recovered by precipitation of the extract with 3 volumes of ethanol.
4. Finally, the insoluble residue is treated with an aqueous 14 % KOH solution at room temperature for 14 h. Then, it is filtered and acidified with glacial acetic

acid to 5–6 pH value. Acidification forms a precipitate which is recovered by centrifugation and corresponds to the fraction of hemicellulose A. The supernatant should be concentrated under reduced pressure, dialyzed against distilled water, and precipitated with 3 volumes of ethanol, resulting in the fraction called hemicellulose B. The alkali insoluble residue is dried in an oven.

Polysaccharides obtained can be quantified by gas chromatography (GC), through FTIR spectroscopy and RMN spectroscopy. If GC is used, weigh 1 mg of hemicellulose to pre-hydrolyze polysaccharides with 500 µL of trifluoroacetic acid 2 mol/L for 4 h at 80 °C; then, allow to dry at room temperature. The following step consists in incubating 100 µg of sample with 250 µL of methanol–HCl 0.5 mol/L anhydrous for 20 h at 80 °C under a nitrogen atmosphere. After drying under nitrogen flow, the methyl glycosides were acetylated and the mixture derivatized was analyzed by gas chromatography. The column suggested is Sil 5-CP (0.53 mm × 50 m fused silica). A flame ionization detector is used with nitrogen (5.5 psi) as carrier gas and the oven temperature program is as follows: start with 150 °C for 10 min and raise the temperature to 200 °C at 0.8 °C/min; hold at 200 °C for 7 min increasing it at 240 °C at 5 °C/min; finally, hold to 240 °C for 20 min.

For spectroscopic analysis see Sects. 2.2.6.1 and 2.2.6.2.

Monoethanolamine Method

Total cellulose is determined by means the monoethanolamine method as it is described by Foyle et al. (2007).

One hundred milliliters of monoethanolamine was added to a dry lignocellulosic sample and located at reflux at 170 °C for 3 h. Then, add 100 mL of water after the sample is cold. 100 mL of the supernatant liquid is decanted into a beaker and the remaining mixture should be filtered through a filtration crucible. The decanted liquid and the filtrate are re-filtered through a cellulose sphere that is formed on the glass crucible during the first filtration. The cellulose should be rinsed with 200 mL of hot water (approximately 60 °C) before washing the solid residue with water in a beaker (to a final volume of 75 mL). In order to bleach cellulose, 10 mL of H_2SO_4 at 10 % v/v and 10 mL of NaOCl (24 g/L) were added. The sample is bleached for 5 min at room temperature. The mixture is filtered through the glass crucible and any residue remaining in the beaker is also rinsed into the crucible using 15 mL of water and 15 mL of H_2SO_4 (0.25 mol/L). The sphere of bleached pulp should be rinsed with 15 mL of cold water followed by 15 mL of 3 % Na_2SO_4. The sample is transferred into a 250 mL beaker using at most 50 mL of water. 5 mL of 6 % w/v Na_2SO_4 is added. The beaker should be covered with a glass watch and incubated in a boiling water bath for 20 min. The appreciation of a pink color at this point indicates the presence of lignin. If this occurs, it is necessary to repeat bleaching. The residue is filtered through the crucible forming a cellulose sphere. Cellulose should be rinsed with 150 mL of boiling water, 50 mL of cold water, 25 mL of 10%w/v acetic acid (very quickly), 50 mL of cold water, 150 mL of boiling water, 75 mL of water

containing two drops of NH_4OH, and finally, with 200 mL of boiling water. The residue should be dried in the furnace at 105 °C and weighed. The cellulose concentration is calculated based on the initial weight of the lignocellulosic sample used.

Hot-Water Extraction

Chen et al. (2013) described the following methodology.

Accurately, weigh 5.0 g of dried and ground biomass and place them within a flask with baffles, add 200 mL of distilled water and place the flask in a bath of water with magnetic stirring (600 rpm and 90 °C). After a period of time of 4 h, the suspension is centrifuged and the pellet is extracted two more times using the same method. The supernatants obtained in each centrifugation are joined and concentrated for vaporization at 50 °C under reduced pressure. Ethanol is added to precipitate polysaccharides and left at 4 °C overnight. The precipitate is centrifuged at 5000 rpm for 10 min and rinsed several times with ethanol. The extraction should be repeated three times.

Quantification

Polysaccharides extracted with hot water can be determined by Chaa method (see Sect. 9.2.2.4.1).

In addition, the yield obtained from the extracted polysaccharides can be determined with the following equation:

$$\text{Yield}(\%) = \frac{W_1}{W_0} \times 100 \tag{9.13}$$

where:

W_1: corresponds to the weight of the extracted polysaccharides.

W_0: it is the weight of the raw material.

Ultrasound and Microwave-Assisted Extraction

This methodology was explained by Huang et al. (2010). The dry biomass is suspended in water and submitted to ultrasound and microwave-assisted extraction. The process with ultrasound takes 60 min at 50 °C while the power used corresponds to 550 W (submitted to ultrasound for a period of 5 and 5 s pause). The microwave treatment is done with 350 W for 10 min in a microwave oven. The insoluble pellet is removed by centrifugation (5000 × *g*, 15 min) and dried under vacuum at 60 °C for 20 h to obtain residue (the humidity content is 12.26 %).

Van Soest method analyses the extraction of cellulose and hemicellulose (see Sect. 9.2.2.1).

High-Performance Liquid Chromatography (HPLC)

Principle

HPLC is a technique used to separate the different compounds within a mixture, in this case, released sugars. It consists in a nonpolar stationary phase (column) and a mobile phase. The stationary phase is usually silica, but depends on the compound to be separated while the mobile phase acts as the carrier sample. The sample in solution is injected into the mobile phase. The components of the solution migrate according to the non-covalent interactions of the compounds with the column. These chemical interactions determine the separation of the sugars contained in the sample. According to the nature of the compounds to be determined, different detectors are taken into account. HPLC is very important because it optimizes the results due to the increase in pressure to which the chromatographic run is subjected; HPLC acts in the stationary phase as an active component complementing the referential liquid mobile phase. The columns used are stainless steel and its length varies between 10 and 30 cm, with internal diameter of 4–10 mm (Orellana and Rogel 2016).

HPLC is mainly used in identifying polysaccharides as it offers various advantages: it is a simple and quick technique; it can be used from microanalytical to industrial scale; allows a continuous record of analysis; can be applied to labile substances due to the low harmfulness; denotes high precision and is not limited to volatile analytes.

Methodology (According to Sluiter et al. 2008a)

Prepare Working Solutions

Prepare a series of calibration standards containing the compounds to be quantified. See literal A and B:

A. *Preparation of standards*:

Perform dilutions in 10 mL volumetric flasks according to each of the compounds to be measured with ultra-purified water (HPLC grade). The concentration of the points of the calibration curve is: 1, 10, 15, 20, 30, and 45 g/L.

B. *Sample preparation*:

Remove 1 mL of hydrolyzate sample. Pour into eppendorf tubes. Centrifuge and then filter with nylon membrane of 0.22 μm porosity. Package in 2 mL HPLC amber vials. Finally, freeze at −10 °C.

Note: Use a micropipette or sterile glass pipette to obtain the extraction of 1 mL from the hydrolyzate obtained from the enzymatic hydrolysis.

HPLC for sugar analysis

(a) Analyze the calibration standards and samples with HPLC using a Sugar Pack or Aminex HPX-87P columns (keep the column off the compartment, which must be stored at room temperature) and a refractive index detector. Be sure to rinse the column. For this, unplug the detector off the column and dispose the waste in a container. Never wash the column with the detector plugged to avoid damages. Run the mobile phase at the same temperature of the method to be used. The flow should be maximum for about 15 min.
(b) After cleansing the column, the patterns are set to run in order to build the calibration curve. Runs should have the same conditions of the samples. Following this, run the sample according to the following conditions:

- Sample volume: 20 μL.
- Mobile phase: water 18.2 MΩ, filtrated for 0.2 μm.
- Flow: 0.5 mL/min (rising gradually to avoid column damage).
- Column temperature: 75 °C.
- Detector temperature: 75 °C.
- Duration: 20 min.
- Detector: refractive index detector (RID).

According to this methodology, the following compounds can be detected under different retention times (Table 9.4):

9.2.2.6 Non-gravimetric Techniques for Polysaccharide Analysis

Infrared Spectroscopy (IR)

Principle

Chemical analysis of lignocellulosic biomass has traditionally been conducted by conventional techniques based on gravimetric methods which result to be rather complex due to the time required to perform the analysis and the demand for large amounts of reagents.

The development of instrumental techniques has enabled analysis to lignocellulosic materials using techniques such as near infrared reflectance spectroscopy (NIRS),

Table 9.4 Retention times for sugars determined by HPLC-RID

Compound	Retention time (min)
Sacarose	7.52
Cellobiose	7.34
Galactose	9.10
Glucose	9.38
Rhamnose	9.80
Xylose	10.28
Fructose	11.69
Arabinose	11.99

analysis that allow certain advantages to gravimetric methods as: the sample requires less preparation; fewer sample-quantity; requires less time in the development of analysis; and, results are reliable (Xu et al. 2013).

IR is a technique that is based on the interaction of electromagnetic radiation with matter. Molecules have frequencies at which they rotate and vibrate (rotation and molecular vibration) and are characteristic of molecular bond types or functional groups present in the sample. This allows to collect information on the structure of a molecule, since molecular-bond information obtained from the chemical compounds of the biomass absorbs specific frequencies of infrared. The most widely used techniques are the Fourier transform infrared spectroscopy (FTIR) and NIRS. NIRS provides structural information by examining the peaks in the spectrum, while FTIR provides information about certain compounds of the plant cell wall through absorbance bands associated with each component.

The NIRS characteristic is that the frequencies of the harmonic movements of molecules are in this subregion comprising the range of wavelength from 700 to 2500 nm or wavenumber from 13,000 to 4000 cm^{-1}. It also provides information on electronic transitions, and overtone bands with symmetric stretching vibration of C–H, O–H, N–H (Cozzolino et al. 2003) are observed. The corresponding electromagnetic radiation in this range interacts with matter. Each molecular bond type has one or more wavelengths. Depending on the molecules and the corresponding bonds, specific joints between atoms vibrate at a certain frequency and each type of these joints absorbs radiation with a specific wavelength. The lengths of unabsorbed waves will be reflected and thus, allowing the construction of a spectrum which is a graphical representation of the vibrational transitions of molecules and bands that appear when the absorption of radiant energy produces changes in the energy of molecular vibration. The final result provides an insight into chemical composition and sample quantification.

For qualitative analysis, the frequencies at which a molecule absorbs radiation are one of its characteristics depending on the links and functional groups present. They also provide and give absorption spectra, while quantification is performed by the intensity of characteristic bands from each functional group associated with the molecule that can determine the concentration.

Table 9.5 describes some absorbance bands in the fundamental infrared where FTIR apply corresponding to functional groups present in the molecular structures of biomass, for example, glycan characteristic bands are used to determine glucose or xylan to determine hemicellulose.

Methodology

The following information describes the methodology employed by Pinzón and Cardona (2008).

In order to carry out FTIR spectra determination, samples of biomass are subjected to a drying process at 50–60 °C to avoid moisture and the presence of broad absorptions due to vibrations around –OH at 3500 and 1400 cm^{-1}, which overlap the existence of other possible bands in those areas. When samples are dry, pulverize

Table 9.5 Absorbance bands in the IR, which are characteristic for certain biomass compounds

Compound	Band generated by	Wave number (cm^{-1})
Cellulose	Hydroxyl groups (–OH) vibration Bond stretching –CH vibrations Asymmetric vibration of C–O–C–, characteristic of carbohydrates	3400–3200 2950–2850 1150
Hemicellulose	Hydroxyl groups (–OH) vibration Vibration of carbonyl groups (–C = O) of the carboxylic acids formed by hydrolysis of the acetyl groups Asymmetric vibration of C-O-C-, characteristic of carbohydrates	3400–3200 1730 1150
Lignin phenylpropane	Vibration bond –C = C– of the aromatic ring	1600–1400
Xyloglucan	Bond $–CH_2$	1370
Glucose	Vibration bond –C–H outside the plane characteristic of ring β-pyranose	1050–890

sample until the particle size is smaller than the wavelength of radiation (less than 2 μm) to avoid the effects of absorption band distortions. The most used technique is the formation of potassium bromide (KBr) plate but other alkali metal halides have been used. Potassium bromide is preferred for its low cost, good transmission and is also ideal for working in the mid-infrared. To prepare the pellet, mix a finely powdered milligram or less of the sample with approximately 100–300 mg of KBr powder (0.2–1 % of concentration in KBr sample). The mixture can be done with a mortar or in a small ball mill. Excessive grinding is not required because KBr will absorb more humidity from the air. Subsequently, press the mixture (between 700 and 1000 kg/cm^2) in a special hydraulic press to obtain a transparent disk. Best results are obtained if the disk is vacuumed to remove entrapped air. Then, the disk is placed on the beam path for spectroscopic examination. The frequency range used is, generally, located between 4000 and 800 cm^{-1}, since the vast majority of classical analytical infrared spectroscopy applications are based on the use of mid-infrared (MIR).

Nuclear Magnetic Resonance Spectroscopy (NMR)

Principle

NMR is the analytical tool that provides greater structural and stereochemical information in affordable time. The technique is nondestructive and has applications in all areas of Chemistry and some of Biology. NMR spectroscopy was developed to study the atomic nuclei and that is the reason why it can be used to determine the structures of organic compounds. This spectroscopic technique can be used only to study atomic nuclei with an odd number of protons or neutrons (or both). This situation occurs in ^{1}H, ^{13}C, ^{19}F, and ^{31}P atoms. Such nuclei are magnetically active, in other words, they possess spin-like electrons because the nuclei have positive charge and a rotational movement on an axis acting as tiny bar magnets.

In the case there is no magnetic field, the nuclear spins are oriented randomly. However, when a sample is placed in a magnetic field, the nuclei with positive spin are oriented in the same direction of the field in a state of minimum energy called state of spin α. Otherwise, the nuclei with negative spin are oriented oppositely to the magnetic field in a higher energy state called spin state β (McMurry 2012).

When a sample containing an organic compound is irradiated briefly by an intense radiation pulse, the nuclei in the spin state α are promoted to spin state β. For a certain field strength value, the energy required to reverse the proton coincides with the radiation, absorption occurs, and a signal is observed. This signal is originated when the nucleus return to their initial state by emitting signals whose frequency depends on the energy difference between the spin states α and β. The NMR spectrometer detects these signals and records them as a graph of frequency *vs* intensity, which is called an NMR spectrum. Thus, the term NMR comes from the fact that the nuclei are in resonance with the applied magnetic field; that is, the nucleus pass from one spin state to another in response to the radiation to which they are subjected.

The presence of many absorption peaks in the NMR spectrum reflects differences in the environment of nuclei and gives a detailed information about the molecular structure. Therefore, the following factors are crucial to interpret a spectrum:

(a) The number of signals indicating the different types of protons in a molecule.
(b) Signal-position denoting certain information about the electronic environment for each type of proton.
(c) Signal-intensity: determines the amount of types of protons present.
(d) Signal-splitting in several peaks, which reports on the environment of a proton in comparison to other nearby protons (Morrison and Boyd 1998).

Methodology

To develop this methodology, the references come from Zhang et al. (2010) and Chaa et al. (2008).

To analyze hemicelluloses fractions, NMR ^{1}H spectrum is performed at 75.5 MHz. Weight and dissolve 25 mg of sample in 1 mL of DMSO-d_6. The test is performed at 353 K and TMS is used as internal standard. For NMR ^{13}C spectrum, a probe 5 mm-BBO (^{13}C 100.67 MHz) is used.

Figure 9.3 explains an example of a NMR^{13}C for pure cellulose and hemicellulose.

9.3 Protein Analysis

There are several techniques for analyzing proteins in biomass. Among the most commonly used techniques is the determination of compounds associated with the cellular concentration of the material. Quantitation is done conveniently with colorimetric methods as the biuret method, Lowry and Bradford. Bluret and Lowry

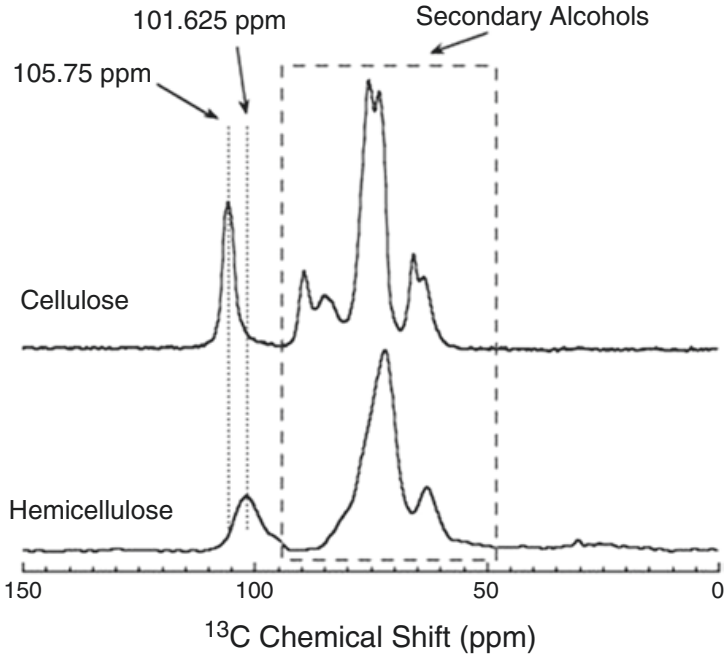

Fig. 9.3 NMR ^{13}C spectrum for pure cellulose and hemicellulose (Cody's 2016)

methods are not recommended due to the low sensitivity of the first one and the interference in the latter. Therefore, the Bradford method is determined to quantify proteins (Child Camacho 2009). In addition to these colorimetric methods, the volumetric method (Kjeldahl method) can also determine proteins.

9.3.1 Bradford Method

9.3.1.1 Principle

The method involves the binding of a dye (Coomassie blue G-250) to proteins. The dye in acidic solution may be found in two colors, one blue and one orange. Total protein binds to the blue dye to form a protein–dye complex with a higher extinction coefficient than the free one (orange dye).

9.3.1.2 Methodology

1. Perform a calibration curve from a serum albumin solution 1 mg/mL using a dilution series of different concentrations from 0.01 to 1 mg/mL.
2. Take 50 mL from each solution and mix with 2.5 mL of Bradford reagent and shake well.
3. Let stand for 3 min and measure the absorbance of the mixture at a wavelength of 595 nm.

4. Determine the standard calibration curve plotting Abs_{595nm} vs standard protein concentration in order to quantify the protein.
5. Determine the protein concentration in the samples from the standard calibration curve and Abs_{595} values. If the concentration is very high (stands outside the range of the standard), dilute the sample or to take a lower aliquot lower.

9.3.2 Kjeldahl Method

9.3.2.1 Principles

The method is based on the destruction of organic matter with concentrated sulfuric acid. In this reaction ammonium sulfate is formed immediately and in excess of sodium hydroxide, ammonia is released. This ammonia is distilled and receiving in sulfuric acid forming ammonium sulfate. The excess of acid is titrated with sodium hydroxide in the presence of methyl red or boric acid. Ammonium borate is formed which is valued with hydrochloric acid.

9.3.2.2 Methodology

The following steps explain the methodology described by Horwitz and Latimer (2005) to characterize protein in biomass:

1. Perform the analysis in duplicate.
2. Perform a control test using an organic substance without nitrogen (sucrose) that is capable of causing reduction of nitric and nitrous derivatives possibly present in the reactants.
3. Weigh to 0.1 mg, 1 g of homogenized simple in a Kjeldahl digestion flask.
4. Add three glass beads, 10 g of potassium or sodium sulfate, 0.5 g of cupric sulfate, and 20 mL of concentrated sulfuric acid.
5. Connect flask to absorption trap containing 250 mL of 15 % w/v sodium hydroxide. The porous disk creates division among fumes into fine bubbles in order to facilitate absorption. To ensure long duration, it is important to cleanse regularly before use. Sodium sulfite deposits are removed with concentrated hydrochloric acid. If sodium hydroxide solution at 15 % w/v added to phenolphthalein (contained in the absorption trap) remains colorless, it must be changed in a rate approx. to 3 analyses.
6. Heat on a blanket and once the solution is clear, let boil 15–20 min more. If the sample tends to foam, add stearic acid or antifoam-silicone-droplets and then, start warming slowly.
7. Cool and add 200 mL of water.
8. Connect the distillation equipment to the flask. Add 100 mL of NaOH at 30 % w/v slowly through the funnel and close the key.

9. Distill at least 150 ml of the solution in a flask submerged at a cooling extreme or collecting tube in:

   (a) 50 mL of sulfuric acid 0.1 mol/L; 4–5 drops of methyl red and 50 mL of distilled water. Ensure an excess of H_2SO_4 so that it can perform the titration. Titrate the excess of acid with NaOH 0.1 mol/L until yellow color appears.
   (b) Fifty milliliters of boric acid at 3 % w/v. Titrate with 0.1 mol/L hydrochloric acid to 4.6 pH using a pH meter calibrated with 4 and 7 pH buffer solutions, or in the presence of the Tashiro indicator to pH value at 4.6.

   It is necessary to check the tightness of distillation apparatus periodically by using 10 mL of a solution of ammonium sulfate 0.1 mol/L (6.6077 g/L), 100 mL of distilled water and 1–2 drops of sodium hydroxide at 30 % to release ammonia. Also, it is important to verify recovery of ammonia by destroying the organic matter from 0.25 g of L(−)-tyrosine.

9.4 Moistures Analysis

9.4.1 Principles

This analytical procedure is based on a traditional convection drying system. However, for biomass suspensions or samples prepared with 10 % (w/w) humidity the method is not recommended. Therefore, use the balance of moisture in these cases.

The gravimetric method determines the moisture content present in the sample through the drying process and by calculating the water percentage *vs* the weight loss due to the elimination of liquid after heating (under normal conditions).

9.4.2 Methodology

Wychen and Laurens (2013) describe this methodology:

Convection Oven Method

(a) Preconditioning of cleansed capsules in a muffle furnace at 575 °C for a minimum of 2 h. This is done to remove any polluting fuel.

Note: Aluminum dishes, pots or porcelain capsules may be used. If aluminum plates are used, precondition them in an oven at 105 °C for 2 h.

(b) Remove caps and cool in a desiccator employing gloves or tweezers. Weigh with an accuracy of 0.1 mg.
(c) Mix the sample and weigh 100 ± 5 mg of biomass in the pre-weighed capsule. Analyze each sample at least in duplicate and include a minimum empty crucible as the control method.

(d) Place the sample in a convection oven at 60 ± 3 °C for at least 18 h.
(e) Remove the sample from the oven and let cool at room temperature in a desiccator.
(f) According to the moisture of the sample, verify the weight of the crucible after 8 h and the weight of the dry sample every 2 h. Place the sample in a convection oven at 60 ± 3 °C again until no variations are greater than 0.01 % i.e. 0.0001 g (constant weight).

9.5 Analysis of Other Compounds

9.5.1 Lipids

9.5.1.1 Principles

It is necessary to carry out derivatization, esterification, and methylation of lipids to realize the characterization of lipids in biomass by GC. These processes are necessary to transform the analyte by chemical reaction in a derivative easier to analyze. The derivatization allows to obtain more volatile species to avoid using too high temperatures that can decompose the analytes in the equipment, or generate unwanted precipitation in the column. Another use of derivatization is to insert a functional group, which increases the resolution and detection into the column, improving the compound separation.

The reactives greatly used for esterification are NaOH, heptane (C7H16), sodium chloride (NaCl), and boron trifluoride (BF3) in methanol. They are used in the esterification of free fatty acids and acylglycerols. *Denote special attention when using BF3 in methanol as it is extremely toxic*.

The most convenient way to convert fatty acids into methyl esters is in basic medium because acid medium may provoke isomers.

The factors that affect directly the quantification of fatty acid methyl esters (FAME) are: incomplete conversion of lipids in FAME and changes in the composition of fatty acids for esterification and formation of compounds that may mistakenly be identified as fatty acids and may directly affect the quantification of FAME.

9.5.1.2 Methodology

The methodology introduced by AOAC Official Methods Ce 2-66 (2009), with the purpose of determining lipids in biomass, sets the guidelines for lipid characterization.

For Fats and Oils (Saponification):

(a) Lipid extraction can be performed through various methods, but the best known is the Soxhlet (see Sect. 9.2.1.1).
(b) Accurate weights are not required. The simple size is necessary to determine the size of balls and amounts of reagents to be applied according to the following Table 9.6.

Table 9.6 Size balls and amount of reagents required according to sample size

Sample (mg)	Balls flat bottom (mL)	NaOH 0.5 mol/L (mL)	BF_3-methanol (mL)
100–250	50	4	5
250–500	50	6	7
500–750	125	8	9
750–1000	125	10	12

(c) If the fat sample extracted from biomass is a solid which adheres to the vessel walls of the recipient, it should be dissolved with methanolic NaOH 0.5 mol/L (see Table 9.6) and heated until dissolved. Following this, pour the solution into the corresponding ball (see Table 9.6) and add three boiling stones. If the fat sample does not stick to the walls, append those to the corresponding ball (see Table 9.6) and add the required amount of NaOH according to Table 9.6.
(d) Bind to a condenser and heat the mixture in a water bath until the fat globules appear in the solution. This step may last 5–10 min.
(e) Add the specific amount of BF_3-methanol and proceed with esterification.

For fat Acids (Esterification)

(a) After adding the specific amount of BF_3-methanol, let boil for 2 min.
(b) Add 2.5 mL of heptane and boil for 1 more minute.
(c) Remove from heat, disassemble the condenser and add about 15 mL of NaCl saturated solution. Plug on the ball and shake vigorously for 15 s while the solution is still warm.
(d) Add enough NaCl saturated solution so that the heptane solution of methyl esters floats within the ball neck. Three phases are formed: organic (top), fat (intermediate), and aqueous (lower).
(e) Transfer heptane solution in the upper layer with a dropper within a test tube and add a small amount of Na_2SO_4 anhydride. Dried heptane solution (top) is transferred to chromatographic vial with a dropper and then is injected directly into the gas chromatograph. If not injected immediately, store for a maximum time of 24 h at −20 °C.

Gas Chromatography

Analyze samples derivatized by GC using two equipments: a cross-linked methyl-siloxane column (length 30 m, 0.32 mm DI) (keep the column off the compartment, it must be stored at room temperature) and a flame ionization detector (FID). Make sure to rinse the column by unpligging the cord that connects to the detector and deposing the waste in a container. Never wash the column with detector 'on' because it can damage the equipment. Run the mobile phase of work at the same temperature of the method to be used. The flow must be at maximum for about 1 h.

The sample runs after cleansing the column and under the following conditions:

- Sample volume: 2 μL
- Gas: helium

- Flow: 1.2 mL/min
- Injector temperature: 250 °C
- Detector temperature: 250 °C
- Temperature ramping: 100 °C × 4 min (3 °C/min), 240 °C × 20 min

With the intention of quantifying the fatty acid methyl esters, a standard series of increasing concentrations should be prepared. Once prepared, inject the same volume of each of the solutions into the chromatograph curve.

In both the chromatogram of each standard solution and from the sample, the peaks correspond to the methyl esters. The retention time of each peak is the same in all chromatograms. However, the area will differ depending on the concentration given to each one.

In order to create the calibration curve a graph with analyte concentration *vs* area of each peak was made according to each concentration. With the equation of the line, calculate the concentration substituting "y" for the peak area corresponding methyl ester in the sample chromatogram. Solve "x" which corresponds to the concentration of methyl ester.

9.5.2 Pectin Analysis

9.5.2.1 Principle

Degradation reactions such as depolymerization and deesterification occur when pectin is extracted of the biomass. Therefore, the conditions of the extraction must be carefully controlled to achieve the desired final properties (Muñoz (2011). Biomass can be characterized by the FTIR technique after extracting pectin from the corresponding biomass, since FTIR allows the identification of functional groups present in the pectin.

9.5.2.2 Methodology

Extraction

Muñoz (2011) described the following methodology for pectin extraction: Weigh samples of 50 g. Use concentrated hydrochloric acid as an acidifying agent of the solutions, since it requires a strong acid to hydrolyze the pectins. The extraction temperature is 93 ± 2 °C. The extraction is performed using Soxhlet equipment in order to maintain process factors without relevant changes because the extraction solvent (acidulated water) usually evaporates at environmental conditions and this could lead to change the proportions of dilution and therefore, the doses of the reagent used.

Filtration and Precipitation

After hydrolysis, pectin is isolated from the exhausted raw material by filtration with a filter cloth and precipitated from the extract with high purity ethanol.

Washing, Sterilizing, and Grinding

The precipitate obtained is rinsed with 50 % ethanol (mixed with distilled water) and pressed to remove soluble impurities. Subsequently, the pectin is dried in an oven for 24 h at an average temperature of 40 ± 3 °C. After drying, grinding is performed for homogenizing the pectin samples to a size of 80 mesh.

FTIR Characterization

This methodology is described by Kacuráková and Wilson (2001). FTIR offers the possibility to determine some functional groups of pectic derivatives in the region of 1900–1500 cm^{-1}.

To carry out the FTIR spectra, samples of extracted pectin are subjected to a drying process at 50–60 °C. Once the samples are moisture-free, they are encapsulated in disks of potassium bromide (KBr). These disks are analyzed in an infrared spectrophotometer. The frequency used for analysis of ester is 1745 cm^{-1} and for carboxylate regions is between 1605 and 1630 cm^{-1}.

9.5.3 Ash Content Determination

9.5.3.1 Principle

The inorganic content in biomass, either structural or removable, must be measured as fraction in the total composition (100 % mqm). The structural ash is inorganic material that is tied to the physical structure of biomass, while the removable ash is inorganic material that can be removed by rinsing. This ash results from soil that is attached to biomass.

Ash is generally the residue of an organic sample that has been incinerated. This is done with the purpose of analyzing the mineral and observing the presence of mineral adulterations. This is a simple method that involves the incineration of biomass at temperature of 400–600 °C. In this process occurs the destruction of all carbonaceous particles.

9.5.3.2 Methodology

The methodology used to determine ash is taken from protocol NREL/TP-510-42622 (Sluiter et al. 2008b).

Mark an appropriate number of crucibles using a porcelain marker and place them in the muffle furnace at 575 ± 25 °C for a minimum of four hours. Remove the crucibles and place them into a desiccator. Record the cooling time. Weigh the crucibles with an accuracy of 0.1 mg and record it.

Place the new crucibles into the muffle furnace at 575 ± 25 °C and dry them to constant weight, which is defined as less than ±0.3 mg of change in the weight for one hour after re-heating the crucible.

Weigh 0.5–2.0 g to the nearest 0.1 mg of the biomass into the tared crucible. Record the weight of the sample. If moisture is the target of the analysis in the sample, then store it in a desiccator until use for ash analysis. Each sample must be analyzed in duplicate at least.

The quantification of ash can be done by using a muffle furnace set at 575 °C or in a muffle furnace with a ramping program.

Determination of Ash Using a Muffle Furnace Set at 575 ± 25 °C

Place the crucible with the sample into a muffle furnace at low heating until smoke stops appearing. Let the crucible cool down before placing it into the muffle furnace. Alternatively, use a muffle furnace with a temperature ramping function to avoid pre-ignition.

Place the crucibles into the muffle furnace at 575 ± 25 °C during 24 ± 6 h. Protect the sample from airflows when handling the crucible to avoid mechanical loss of sample.

Carefully, remove the crucible from muffle furnace and place it into a desiccator. Cool the crucible for a specific amount of time, equal to the time of initial cooling of all crucibles. Weigh the crucibles and ash to the nearest 0.1 mg and record the weight.

Place the new crucibles into the muffle furnace at 575 ± 25 °C and dry them to constant weight, which is defined as less than ±0.3 mg of change in the weight upon 1 h after re-heating the crucible.

Determination of Ash into Muffle Furnace Equipped with a Ramping Program

Set the heating rate in the muffle furnace according to the following scheme:

- Ramp to room temperature at 105 °C
- Hold at 105 °C for 12 min
- Ramp to 250 °C with ratio 10 °C/min
- Hold at 250 °C for 30 min
- Ramp to 575 °C at 20 °C/min
- Hold at 575 °C for 180 min
- Allow temperature to drop to 105 °C

- Hold at 105 °C until samples are removed

Place the crucibles into muffle furnace and begin the ramping program. Protect the sample from airflows when handling the crucible to avoid mechanical loss of sample.

Carefully remove the crucible from muffle furnace and place it into a desiccator. Cool the crucible for a specific amount of time, equal to the time of initial cooling of all crucibles. Weigh the crucibles and ash to the nearest 0.1 mg and record the weight.

Place the new crucibles into the muffle furnace at 575 ± 25 °C and dry them to constant weight, which is defined as less than ±0.3 mg of change in weight for an hour after re-heating the crucible.

References

AOAC (1995) AOAC official method 973.18. Fibre (acid detergent) and Lignin in animal feed. In: AOAC (ed) AOAC official methods of analysis (vol 1, Chap. 4), 16th edn. AOAC, Rockville, MD, pp 20–21

AOAC (2002) AOAC official method 2002.04. Amylase-treated neutral detergent fiber in feeds. In: AOAC (ed) AOAC official methods of analysis, vol 85, number 6, 1st edn. AOAC, Rockville, MD, pp 20–21

AOAC (2009) AOAC official methods Ce 2–66. Preparation of methyl esters of fatty acids. Official methods 6a. AOCS, Urbana

ASTM D1110-84 (2013) Standard test methods for water solubility of wood. ASTM International, West Conshohocken, PA. www.astm.org

Attard TM, Rob Mcelroy C, Rezende CA, Polikarpovc I, Clarka JH, Hunt AJ (2015) Sugarcane waste as a valuable source of lipophilic molecules. Ind Crops Prod 76:95–103

Bhattacharya D, Germinario LT, Winter WT (2008) Isolation, preparation and characterization of cellulose microfibers obtained from bagasse. Carbohydr Polym 73:371–377

Ballesteros I, Oliva JM, Saez F, Ballesteros M (2001) Ethanol production by simultaneous saccharification and fermentation of olive oil extraction. Appl Biochem Biotechnol 91(93):237–252

Bian J, Peng F, Peng X-P, Xiao X, Peng P, Xu F (2014) Effect of [Emim]Ac pretreatment on the structure and enzymatic hydrolysis of sugarcane bagasse cellulose. Carbohydr Polym 100:211–217

Boerjan W, Ralph J, Baucher M (2003) Lignin biosynthesis. Annu Rev Plant Physiol Plant Mol Biol 54:519–546

Buranov AU, Mazza G (2008) Lignin in straw of herbaceous crops. Ind Crops Prod 28:237–259

Cardoen D, Joshi P, Diels L, Sarma PM, Pant D (2015) Agriculture biomass in India: Part 1. Estimation and characterization. Resour Conserv Recy 102:39–48

Cataño Rueda EH (2009) Obtención y caracterización de nanofibras de celulosa a partir de desechos agroindustriales. Tesis Ingeniería Química, Facultad de Minas, Escuela de Procesos y Energía, Universidad Nacional de Colombia, Medellín, Colombia, p 13. http://www.bdigital.unal.edu.co/920/1/1017137266_2009.pdf

Chaa L, Joly N, Lequart V, Faugeron C, Mollet JC, Martin P, Morvan H (2008) Isolation, characterization and valorization of hemicelluloses from Aristida pungens leaves as biomaterial. Carbohydr Polym 74:597–602

Chen J, Lai P, Shen H, Zhen H, Fang R (2013) Effect of extraction methods on polysaccharide of Clitocybe maxima stipe. Adv J Food Sci Technol 5(3):370–373

Cody's G (2016) Solid state NMR facility [Figure] recovery from. https://www.gl.ciw.edu/static/users/gcody/nmr.html
Cozzolino D, Fassio A, Fernández E (2003) Use of near infrared reflectance spectroscopy to analyze corn silage quality. Agric Téc 64(3):387–393
Del Río JC, Lino AG, Colodette JL, Lima CF, Gutierrez A, Martínez AT, Lu F, Ralph J, Rencoret J (2015) Differences in the chemical structure of the lignins from sugarcane bagasse and straw. Biomass Bioenerg 81:322–338
Fengel D, Wegener G (1984) Wood: chemistry, ultrastructure, reactions. De Gruyter, Berlin, p 613
Foyle T, Jennings L, Mulcahy P (2007) Compositional analysis of lignocellulosic materials: evaluation of methods used for sugar analysis of waste paper and straw. Bioresour Technol 98:3026–3036
García Sánchez A, Ramos Martos N, Ballesteros E (2005) Estudio comparativo de distintas técnicas analíticas (espectroscopía de NIR y RMN y extracción mediante Soxhlet) para la determinación del contenido graso y de humedad en aceitunas y orujo de Jaén. Grasas Aceites 56(3):220–227
Godin B, Agneessens R, Gerin PA, Delcarte J (2011) Composition of structural carbohydrates in biomass: precision of a liquid chromatography method using a neutral detergent extraction and a charged aerosol detector. Talanta 85:2014–2026
Golander E (2011) Characterization and methods for extraction of extractives in spent sulphite liquor. Master of science thesis, Department of Chemical and Biological Engineering, Chalmers University of Technology, p 8. http://publications.lib.chalmers.se/records/fulltext/142119.pdf
Higuchi T (1985) Lignin biosynthesis. In: Higuchi T (ed) Biosynthesis and biodegradation of wood components. Academic, Orlando, FL, pp 114–160
Horwitz W, Latimer GW (2005) Chapter 33: official methods of analysis of AOAC international, 18th edn. AOAC International, Gaithersburg, MD
Huang SQ, Li JW, Wang Z, Pan HX, Chen JX, Ning ZX (2010) Optimization of alkaline extraction of polysaccharides from Ganoderma lucidum and their effect on immune function in mice. Molecules 15:3694–3708
Kacuráková M, Wilson RH (2001) Developments in mid-infrared FT-IR spectroscopy of selected carbohydrates. Carbohydr Polym 44:291–303
Luque de Castro MD, García-Ayuso LE (1998) Soxhlet extraction of solid materials: an outdated technique with a promising innovative future. Anal Chim Acta 369:1–10
Luque de Castro MD, Priego-Capote F (2010) Soxhlet extraction: past and present panacea. J Chromatogr A 1217:2383–2389
Martín Lara, María Angela. Caracterización y aplicación de biomasa residual a a la eliminación de metales pesados [in line]. Tesis doctoral Ciencia y Tecnología del Medio Ambiente. Granada. Universidad de Granada. Facultad de Ciencias, 2008. 424 p. [consulta: 20 mayo de 2015]. In: http://hera.ugr.es/tesisugr/17514629.pdf
McMurry J (2012) Química orgánica 8a edición. Cengage Learning, México DF
Morrison RT, Boyd RN (1998) Química Orgánica. Addison Wesley Longman, México
MUÑOZ, F. J. Extracción y caracterización de la pectina obtenida a partir del fruto de dos ecotipos de cocona (Solanum sessiliflorum), en diferentes grados de madurez; a nivel de planta piloto. [In line]. Tesis Maestría en Ingeniería Agrícola. Bogotá, Colombia: Universidad Nacional de Colombia, Facultad de Ingeniería, Departamento de Ingeniería Civil y Agrícola, 2011. 18 p. [Consulta: 06 de abril de 2016] In:http://www.bdigital.unal.edu.co/4006/1/822093.2011.pdf
Niño Camacho LR (2009) Implementación de diferentes técnicas analíticas para la determinación de biomasa bacterinas de cepas *Psudomonas putida* biodegradadoras de fenol. [en línea]. Tesis Química. Facultad de Ciencias, Escuela de Quíimica, 12Universidad Industrial de Santander, Santander, Colombia, p 19. http://repositorio.uis.edu.co/jspui/bitstream/123456789/363/2/131320.pdf
Oliva Domínguez JM (2003) Efecto de los productos de degradación originados en la explosión por vapor de biomasa de chopo sobre *Kluyveromyces marxianus*. Tesis doctoral, Madrid, p 166. http://biblioteca.ucm.es/tesis/bio/ucm-t26833.pdf

Oliva Dominguez, J. Miguel. Efecto de los productos de degradación originados en la explosión por vapor de biomasa de chopo sobre Kluyveromyces marxianus. [en línea]. Tesis doctoral. Madrid, 2003. 166 p. [consulta: 5 de marzo de 2015]. Disponible en: http://biblioteca.ucm.es/tesis/bio/ucm-t26833.pdf

Orellana V, Rogel A (2016) Cromatografía de líquidos de alta resolución. Unidad Académica de Ciencias Químicas de la Salud, Universidad Técnica de Machala. https://issuu.com/toxicologia6/docs/hplc.docx_29f51bdf70a77d

Pérez MJ, Quishpi JA (2014) Evaluación cuantitativa de la producción de biodiesel de microalgas de lagunas de tratamiento de agua residual. Tesis Ingeniería Civil. Facultad de ingeniería, Escuela de Ingeniería Civil, Universidad de Cuenca, Cuenca, Ecuador, p 111. http://dspace.ucuenca.edu.ec/bitstream/123456789/20934/3/TESIS.%20PDF.pdf

Pinzón ML, Cardona AM (2008) Caracterización de la cáscara de naranja para su uso como material bioadsorbente. Bistua Rev 6(1):28–37

Sarkanen KV, Ludwig CH (1971) Lignins: occurence, formation, structure and reactions. Wiley Interscience, New York, NY, p 916

Sluiter A, Hames B, Ruiz R, Scarlata C, Sluiter J, Templeton D (2008a) Determination of extractives in biomass. NREL/TP-510-42619. National Renewable Energy Laboratory, Golden, CO

Sluiter A, Hames B, Ruiz R, Scarlata C, Sluiter J, Templeton D, Crocker D (2008b) Determination of structural carbohydrates and lignin in biomass. NREL/TP-510-42618. National Renewable Energy Laboratory, Golden, CO

Sluiter A, Hames B, Ruiz R, Scarlata C, Sluiter J, Templeton D (2008c) Determination of ash in biomass. NREL/TP-510-42622. National Renewable Energy Laboratory, Golden, CO

Szcrbowski D, Pitarelo AP, Filho AZ, Pereira L (2014) Sugarcane biomass for biorefineries: comparative composition of carbohydrate and non-carbohydrate components of bagasse and straw. Carbohydr Polym 114:95–101

Turrel FM, Fisher PL (1942) The proximate chemical constituents of citrus woods, with special reference to lignin. Plant Physiol 17(4):558–581

Pilnik W, Voragen AGJ (1993) Enzymes in food processing, 3rd edn. Copyright 1993 by Academic. 363 3 64. Capítulo 1: pectic enzymes in fruit and vegetable juice manufacture. p 363–392

Wychen SV, Laurens LM (2013) Detemination of total solids and ash in alga biomass. Tech Rep NREL/TP:5100–60956

Xu F, Yu J, Tesso T, Dowell F, Wang D (2013) Qualitative and quantitative analysis of lignocellulosic biomass using infrared techniques: a mini-review. Appl Energ 104:801–809

Zhang J, Deng H, Lin L, Sun Y, Pan C, Liu S (2010) Isolation and characterization of wheat straw lignin with a formic acid process. Bioresour Technol 101:2311–2316